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This project is desinged to provide a better understanding of rattlesnake neurotoxin structure, mechanism of action, immunology and molecular biology. Toward this end we have sequenced the basic subunit of Mojave toxin and the B-chain of crotoxin, which completes the amino acid sequence studies on these two proteins, except for determination of their disulfide bond arrangements. These arrangements should be elucidated from x-ray analysis of Mojave toxin crystals currently in progress with Keith Ward. Two myotoxins from the venom of Bothrops asper have been sequenced. One is a potent myotoxic, phospholipase A_2 that lacks neurotoxicity--the first such protein to be sequenced--and the second is a lysine-49 phospholipase A_2 with key amino acid differences from active phospholipases. Notexin isoforms (scutoxins A and B) have been isolated and partially characterized from Notechis scutatus venom. We further demonstrated that not all Notechis subspecies venom contain notexin and presynaptic isoforms. Iodinated crotoxin has been used to demonstrate high affinity, specific binding sites to guinea pig brain synaptosomes. (See reverse for con

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19. Abstract continued

Work suggests that a specific receptor exists that is also recognized by several other pre-synaptic neurotoxins. Studies done on the phrenic nerve-hemidiaphragm with representative pre-synaptic neurotoxins, indicate that these toxins may insert into the presynaptic membrane, but not penetrate through it. Immunological experiments designed to look at crotoxin epitopes suggest four linear antigenic regions are present in the basic subunit, but none of these generate lethality neutralizing antibodies in rabbits. Our neutralizing monoclonal antibody does not recognize a linear epitope. cDNA clones for both subunits of Mojave toxin have been isolated and sequenced by Leonard Smith. Attempts to get them expressed in E. coli systems are in progress in our laboratory. We have also isolated genomic clones for both subunit genes of Mojave toxin and are currently subcloning these proteins in preparation for sequencing.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

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JJC In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

James J. Vacca 9/4/90

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INTRODUCTION

NATURE OF THE PROBLEM

This contract was designed to provide a better understanding of rattlesnake neurotoxin structure, mechanism of action, immunology and molecular biology. When written in 1988, evidence had accumulated suggesting great similarities between crotoxin, Mojave toxin from C. s. scutulatus, concolor toxin from C. v. concolor, and vegrandis toxin from C. vegrandis in both structure and function (Aird and Kaiser, 1985; Kaiser et al., 1986; Kaiser and Aird, 1987). Our recent studies and those of others (Henderson and Bieber, 1986) have convincingly demonstrated the structural and functional similarities of perhaps all rattlesnake neurotoxins. This group of presynaptic neurotoxins consist of a moderately toxic basic phospholipase A_2 and a non-covalently associated non-toxic, acidic subunit. The acidic subunit is required for full toxicity, but has no other identified function. Binding experiments suggest that the acidic subunit functions as a "chaperone", improving their targeting of the basic subunit by preventing non-specific adsorption of the complex (Chang, 1985). Crotoxin's acidic subunit is presumably released upon binding of the basic subunit at the membrane target site. It is unclear how crotoxin identifies the proper target in vivo and what promotes dissociation of the subunits. Studies proposed with the phospholipases A_2 from B. asper will determine whether monomeric, basic phospholipases A_2 can associate with the acidic subunit of crotoxin and if they can, determine whether there is any indication of an induced "neurotoxin action" in a normally non-neurotoxic molecule. Sequencing of these molecules will also expand our basic, phospholipase A_2 data base and make available a venom derived monomeric, lipase for trial crystallization studies. Sequencing of the basic subunit of Mojave toxin is also proposed in this application, to facilitate our x-ray structural determination of this molecule. Finally, we propose to prepare highly purified acidic and basic subunit of Mojave toxin, as well as F_{ab} fragments of our neutralizing monoclonal antibodies for crystallization trials. All crystallization trials and x-ray diffraction studies are being carried out in the laboratory of Dr. Keith Ward (Naval Research Laboratories). These structural studies will further enhance our understanding of the higher-ordered structure of the rattlesnake neurotoxins, their subunits, and related presynaptic neurotoxins.

Snake presynaptic toxins such as crotoxin, are known to block neuromuscular transmission by inhibiting the release of acetylcholine

from the presynaptic nerve terminal, but the detailed molecular mechanisms have not been established (Chang, 1985). A better understanding of this mechanism is ultimately essential if we are to rationally develop different materials that interfere with the neurotoxin's actions. It has been proposed by Bon et al. (1988) that the negatively charged phospholipids are a possible target for crotoxin. The presence of other phospholipases A_2 with little or no neurotoxicity, suggests that there are specific binding sites of high affinity for neurotoxic phospholipases A_2 on neural membranes, and no such sites exist for non-neurotoxic lipases (Rehm and Betz, 1982, 1984; Tzeng et al., 1986). In our studies on the mechanism of action, we proposed several approaches to probe crotoxin's neurotoxic mechanism. One involved a search and characterization of a receptor on synaptosomal membrane using ^{125}I-labeled crotoxin and a cross-linking photoaffinity probe. A preliminary report by Tzeng et al. (1986) has described such a receptor, but no characterization was done. A similar membrane protein was reported earlier by Rehm and Betz (1983) for ß-bungarotoxin. We were interested in searching for such a receptor moiety, and if found determine its make up, molecular weight, association characteristics, etc. We were also interested to determine whether the receptor was associated with a higher-ordered binding complex as found for ß-bungarotoxin in chick brain membranes (Rehm and Betz, 1984). We are attempting to visualize the interaction of crotoxin with nerve terminals in a series of in vivo and in vitro experiments using ^{125}I-labeled crotoxin and electron microscope autoradiographic procedures. These studies may provide insight on whether crotoxin is internalized and if so what is the mechanism of internalization (see recent papers by Black and Dolly, 1986a and b). Finally, bioluminescence methodology described by Israel and Lesbats (1987) have been applied to the measurement of acetylcholine released from isolated synaptosomes. These new techniques may permit for the continuous detection of acetylcholine release in physiological solutions and greatly enhance the usefulness of synaptosomes and tissue culture cells as an assay system for examining the effects of different toxins on acetylcholine release.

Our preparation (Kaiser and Middlebrook, 1988a) of a neutralizing monoclonal antibody against the basic subunit of crotoxin provided a tool to attempt to identify a synthetic peptide fragment that could be used as a non-toxic, synthetic vaccine (Walter, 1986) against crotoxin toxicity. Also, the delineation of the full profile of continuous antigenic sites of the basic subunit of crotoxin using polyclonal antisera can soon be correlated with their conformational locations, based on the three-dimensional studies of the crotoxin homolog, Mojave toxin by Keith Ward's

laboratory (Norden et al., 1987). These, and related immunological studies, should give us a better understanding of crotoxin and presynaptic neurotoxin immunology.

As noted before, little is known about the biosynthesis of crotalid neurotoxins, or any venom proteins found in reptiles. We have been successful in preparing cDNA and genomic libraries of Crotalus s. scutulatus in lambda phage in collaboration with Dr. Leonard Smith. cDNA clones for both subunits of Mojave toxin have been isolated and sequenced in Leonard Smith's laboratory. We have identified and are currently working with genomic clones containing both subunits of Mojave toxin. These studies will permit manipulation and selective alterations of the crotoxin gene. In addition to the oligonucleotide-directed site-specific mutagenesis studies, designed to generate a non-toxic, antigenic crotoxin homolog, there are a number of questions concerning the toxin that need answering.

BACKGROUND

In earlier work we were concerned with sequencing studies of the basic and acidic subunit of crotoxin. We completed the sequence of the basic subunit, and have published those results (Aird, Kaiser, Lewis, and Kruggel; 1986 and 1987). Two of the three acidic subunit chains, and 24-residues of the carboxyl-terminus of the third chain were sequenced (Aird, Kaiser, Lewis, and Kruggel, 1985). Repeated attempts to sequence the blocked, amino-terminal end of the B-chain by conventional methods were unsuccessful. In 1987, we initiated a collaboration with Dr. Donald F. Hunt at the University of Virginia, whose laboratory employs tandem mass spectrometry for determining amino acid sequences in proteins. He was recently able to provide us with the amino-terminus sequence of the B-chain as shown below.

pE-E-D-G-E-I-V-C-G-E-D-D-D-P-C-...

A manuscript describing this work is in press (Aird, Yates, Martino, Shabanowitz, Hunt, and Kaiser, 1990). Sequencing of the three peptides present in the acidic subunit, two of which are blocked by pyroglutamate, represents a significant contribution. Others have unsuccessfully attempted to sequence the acidic subunit for the past fifteen years.

Structural studies on crotoxin, its subunits, and related toxins, have been completed. We examined the spectral properties of four presynaptic neurotoxins from the venoms of C. d. terrificus, C. vegrandis, C. s. scutulatus, and C. v. concolor. These and their subunits were examined by circular dichroism, deconvolution Fourier-transform infrared, and fluorescence spectroscopy. This work has now been published (Aird, Steadman, Middaugh, and Kaiser, 1989) which suggested that in general

the isolated subunits were decreased slightly in α-helix, while they were increased in ß-sheet structure, relative to intact toxins. We initially concluded from these results that major conformational changes occurred in individual subunits upon formation of the dimeric toxins. Intact crotoxin, however, when exposed to urea, yields spectra that are virtually identical to control intact crotoxin. These findings suggest that the enhanced fluorescence exhibited by the isolated subunits, as well as the secondary structural changes in α-helix and ß-sheet, are artifacts resulting from irreversible structural changes that occur during subunit isolation by urea ion-exchange chromatography.

We examined the venom from the Great Basin rattlesnake (C. v. lutosus), the Uracoan rattlesnake (C. vegrandis), and the Western diamondback rattlesnake (C. atrox), as well as Western diamondback-Mojave rattlesnake (C. s. scutulatus) hybrids, for crotoxin-like neurotoxins. One publication has resulted on the Uracoan study (Kaiser and Aird, 1987); one on the Great Basin rattlesnake (Aird, Seebart, and Kaiser; 1988), and one on the Western diamondback (Aird, Thirkhill, Seebart, and Kaiser; 1989). We also examined the myotoxin fraction from C. v. concolor, as a result of earlier indications that there were some sequence homologies between these smaller peptides and the basic subunit of crotoxin, as well as the existence of myotoxin isoforms. A publication describing these results has been published (Ownby, Aird, and Kaiser, 1988). Dr. Keith Ward has been conducting x-ray crystallography studies on the crotoxin homolog, Mojave toxin, which we purified and provided to him. A preliminary report has appeared on this work (Norden, Ward, Kaiser, and Aird; 1987) which is continuing.

Modification studies on purified crotoxin were carried out in attempts to identify structural requirements for toxicity. We have approached this problem chemically and immunologically. Four different chemical cross-linkers and one Lys-specific monofunctional reagent have been used in crotoxin modification studies. We finally concentrated on the most promising of these, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide. We determined that cross-linked crotoxin was non-toxic at i.v. injections up to 2-3 µg/g in mice and concluded that loss of toxicity was due to either subunit cross-linking or modification of essential residues (see BODY and reference by Lennon, Plummer, and Kaiser; 1990).

Our immunological studies have involved polyclonal antisera raised to crotoxin, its subunits, and crotoxin homologs as described in the publication by Kaiser, Middlebrook, Crumrine, and Stevenson (1986). Further, in collaboration with Dr. John Middlebrook, we have isolated four monoclonal antibodies raised to the basic subunit of crotoxin. One is a potent neutralizer of crotoxin's lethality and phospholipase A_2 activity as

described in the following publications (Kaiser and Middlebrook, 1988a and b). Middlebrook's laboratory has now prepared anti-idiotype monoclonal antibodies against the neutralizing monoclonal and are examining their potential as crotoxin immunogens. We also examined these for possible phospholipase activity, but found none.

We conducted preliminary experiments using guinea pig brain synaptosomes and ^{3}H-choline release measurements to monitor responses to crotalid neurotoxins. Existing assays and procedures have proved to be more extensive and involved than originally anticipated. New methodology, employing luminometry was examined, but in our hands has not been satisfactory with brain synaptosomes. We are continuing to examine brain synaptosomes and ^{3}H-choline uptake as a system for studying presynaptic neurotoxin action in vitro.

Dr. Lance Simpson (Jefferson Medical College) has examined crotoxin for its neuromuscular blocking properties on the isolated phrenic nerve-hemidiaphragm preparation from the mouse. He found the toxin produces concentration-dependent paralysis of transmission with a high temperature dependence. Our crotoxin-neutralizing monoclonal antibody at equimolar concentration, abolishes the toxicity if premixed prior to addition to tissues. If antibody is added to tissues after the toxin has become bound, it no longer affords protection. One of our non-neutralizing monoclonal antibodies at equimolar concentrations to crotoxin, does not delay onset of neuromuscular blockage. A publication describing this work has now appeared (Trivedi, Kaiser, Tanaka and Simpson, 1989).

Work relating to the cloning of the genes for the crotalid presynaptic neurotoxin had several false starts. In collaboration with Dr. Leonard Smith (USAMRIID), we have now successfully constructed both cDNA and genomic libraries of Crotalus s. scutulatus. Acidic and basic subunit clones have been identified in both libraries. Smith has now sequenced three acidic and four basic subunit clones from the cDNA library. We are attempting to sub-clone the genomic clones of both subunits in preparation for sequencing.

PURPOSE OF THE PRESENT WORK

This is a multifaceted approach involving a detailed examination of rattlesnake neurotoxins will provide us with a better understanding of these molecules. At the conclusion of this contract, we should know (1) more about the primary, secondary, and tertiary structure of rattlesnake neurotoxins and non-toxic phospholipase A_2 molecules; (2) whether crotoxin interacts with a membrane binding receptor and something about that receptor; (3) whether crotoxin is internalized at the motor neurons

and the mechanism of that internalization; (4) whether an in vitro system for monitoring continuous acetylcholine release from synaptosomes is feasible; (5) the full profile of continuous antigenic sites on the basic subunit of crotoxin and possibly the epitope recognized by our neutralizing monoclonal antibody; (6) whether the neutralizing monoclonal antibody F_{ab}-antigen complex is crystallizable; and (7) whether non-toxic crotoxin antigen can be generated by site-specific mutagenesis.

The resulting data will allow possible insights into methods of defense against rattlesnake neurotoxins, a better understanding of their basic chemistry, mechanism of action, and therapies; and a possible in vitro model system for presynaptic neurotoxin assay and screening of potential therapeutic compounds.

METHODS OF APPROACH

a. Structure. 1. Determine the disulfide bond arrangement in the basic subunit of crotoxin using a combination of chemical and mass spectrometer methods. **2.** Determine the primary sequence of the basic subunit of Mojave toxin. **3.** Determine the primary sequence of the the monomeric, basic phospholipase A_2 from Bothrops asper, and examine its ability to associate with the acidic subunit of crotoxin. We will also provide Dr. Keith Ward (Naval Research Laboratories) with sufficient purified material of this basic phospholipase for crystallization experiments. **4.** Prepare highly purified acidic and basic subunits of Mojave toxin for crystallization trials by Ward's laboratory.

b. Mechanism of Action. 1. Prepare ^{125}I-labeled crotoxin which retains its neurotoxicity and phospholipase activity. Use this material in examining (i) its binding to synaptosomal membranes, (ii) cross-linking to specific membrane components, (iii) brain synaptic membranes for high affinity crotoxin binding sites, and (iv) crotoxin's interaction with nerve terminals, using electron microscopic autoradiography. **2.** Employ new bioluminescence techniques to monitor continuous acetylcholine release from brain synaptosomes in vitro, in the absence and presence of purified presynaptic neurotoxins.

c. Immunology. 1. Examine the full profile of continuous antigenic sites on the basic subunit of crotoxin and attempt to identify the epitope recognized by our neutralizing monoclonal antibody. **2.** Prepare highly purified F_{ab} fragments of our neutralizing monoclonal antibody for crystallization attempts of the F_{ab}-antigen complex by Keith Ward.

d. Continue our work on the **molecular biology** of crotoxin, directed to the eventual production of a non-toxic crotoxin antigen by site-specific mutagenesis.

BODY OF REPORT

METHODS, RESULTS, and DISCUSSION

a. STRUCTURE

1. MOJAVE TOXIN. The complete sequence of the basic subunit of Mojave toxin from the venom of the Mojave rattlesnake (Crotalus s. scutulatus) was determined. It was shown to have great similarity to the basic subunits of related toxins from the venoms of the South American and midget faded rattlesnakes. Experimental details may be found in the recently published paper by Aird, Kruggel and Kaiser (1990).

2. CROTOXIN. The B-chain of the acidic subunit of crotoxin proved refractory to Edman degradation. When subjected to sequence analysis using tandem mass spectrophotometry, pyroglutamate was found at the amino-terminal end, even though earlier attempts to deblock with pyroglutamate aminopeptidase were unsuccessful. The B-chain contained 35 amino acids and showed 91% amino acid identity with the corresponding segment of Mojave toxin, a homologous neurotoxin from Crotalus scutulatus scutulatus (Bieber et al., 1990). The sequence of the last 24 residues of the B-chain is consistent with that previously published (Aird et al., 1985) except at position 20, where Edman degradation gave glycine and mass spectrometry gave glutamic acid. Additional details on the determination of the B-chain sequence using mass spectrometry may be seen in the reference by Aird, Yates, Martino, Shabanowitz, Hunt, and Kaiser (1990).

3. BOTHROPS ASPER PHOSPHOLIPASES AND PHOSPHOLIPASE-LIKE MOLECULES. A myotoxic, basic phospholipase A_2 (pI>9.5) with anticoagulant activity has been purified from the venom of Bothrops asper, and its amino acid sequence determined by automated Edman degradation. It is distinct from the B. asper phospholipase A_2 known as myotoxin I [Lomonte and Gutierrez (1989)], but cross-reacts with myotoxin I rabbit antisera, suggesting that the proteins are closely related isoforms. To our knowledge, this is the first myotoxic phospholipase to be sequenced that lacks presynaptic neurotoxicity (i.v. LD_{50} ~8 μg/g in mice). The protein appears to exist as a monomer, contains 122 amino acids and fits with subgroup IIA of other sequenced phospholipase A_2 molecules. Its primary sequence shows greatest identity with ammodytoxin B (67%), a phospholipase A_2 presynaptic

neurotoxin from Vipera ammodytes ammodytes venom. Hydropathy profiles of B. asper phospholipase and the ammodytoxins also show great similarities. In contrast, even though the amino acid sequence identities between B. asper phospholipase and the basic subunit of crotoxin remain high (64%), their hydropathy profiles differ substantially. Domains and residues that may be responsible for neurotoxicity are discussed. Experimental details may be found in the reference of Kaiser, Gutierrez, Plummer, Aird, and Odell (1990).

A second phospholipase-like molecule from the venom of B. asper has also been recently sequenced. This protein, a basic, dimeric myotoxic protein, myotoxin II, has a similar molecular weight and is immunologically cross-reactive with antibodies raised to previously isolated B. asper phospholipases A_2, except that it shows only 0.1% of the phospholipase activity against L-α-phosphatidylcholine in the presence of Triton X-100. Its 121 amino acid sequence, determined by automated Edman degradation, clearly identifies it as a Lys-49 phospholipase A_2. Key amino acid differences between myotoxin II and phospholipase active proteins in the Ca^{++}-binding loop region, include Lys for Asp-49, Asp for Tyr-28, and Leu for Gly-32. The latter substitution has not previously been seen in Lys-49 proteins. Other substitutions near the amino terminus (Leu for Phe-5 and Gln for several different amino acids at position 11) may prove useful for identifying other Lys-49 proteins in viperid and crotalid venoms. Myotoxin II shows greater sequence identity with other Lys-49 proteins from different snake venoms (Agkistrodon piscivorus piscivorus, Bothrops atrox and Trimeresurus flavoviridis) than with another phospholipase A_2 active Asp-49 molecule isolated from the same B. asper venom. This work demonstrates that phospholipase activity *per se*, is not required in phospholipase molecules for either myotoxicity or edema inducing activities. Experimental details may be found in the manuscript by Francis, Gutierrez, Lomonte, and Kaiser (1990).

4. NOTECHIS. Venoms from Notechis scutatus scutatus, Notechis ater serventyi, Notechis ater humphreysi, and Notechis ater ater were compared using gel filtration, polyacrylamide gel electrophoresis, and western blotting. All venoms gave slightly different elution profiles on a Superose 12 gel filtration column. When examined by SDS-PAGE and western blotting, all venoms appeared to have notexin like proteins except for N. a. serventyi. N. a. serventyi lacked proteins migrating in the notexin molecular weight range, showed no reactivity toward a monoclonal antibody that reacted strongly with notexin in western blot analysis, and had twice the LD_{50}-value of the other three venoms. Experimental details

may be found in the paper by John and Kaiser (1990).

5. NEW TOXINS (SCUTOXINS) FROM THE VENOM OF THE COMMON TIGER SNAKE (NOTECHIS SCUTATUS SCUTATUS). During our preparation of notexin and notechis II-5 under contract DAMD 17-86-C-6061, we isolated a protein which was less basic than either notexin or notechis II-5, but was as toxic as notexin. These new toxins are described in a publication by Francis, John, Seebart and Kaiser (1990), which also provides an alternative procedure for the isolation of highly purified notexin and notechis II-5. The new toxins, scutoxin A and B represent two isoforms of a new toxic protein from the venom of the Australian Tiger Snake, Notechis scutatus scutatus. Both isoforms, of apparent mol. wt. 13,000, are less basic than either notexin or notechis II-5. They both have similar i.v. LD_{50}-values in mice of ca. 0.006 µg/g, and phospholipase activities of about 136 µmoles fatty acid released min^{-1} mg^{-1} at 37° when acting on phosphatidyl choline in the presence of Triton X-100. Toxicities of the scutoxins are the same as notexin and about seven-times more potent than notechis II-5. ELISAs and western blot analyses indicate that the new toxins are immunologically similar to notexin and notechis II-5, with phospholipase activities falling between these latter two proteins. When crude venom is initially passed over a gel filtration column, each scutoxin isoform co-elutes in a different fraction with notexin. Gel filtration experiments using purified samples of notexin and scutoxin have failed to demonstrate any evidence for the formation of higher molecular weight protein complexes. Peptide mapping suggests the presence of five glutamate residues in one of the protein isoforms. These findings, together with the high toxicity and active phospholipase levels, demonstrate that the new proteins are not the previously reported non-toxic and enzymatically inactive notechis II-1. Recent unpublished sequencing results in collaboration with Dr. Jim Schmidt (USAMRIID), indicate that the first 28 amino terminal residues of scutoxin A and the first 36 residues of scutoxin B are the same as notexin, which clearly identifies these toxins as notexin isoforms. The combination of gel filtration on Sephacryl S-200 and cation-exchange chromatography used to isolate the scutoxins also permits recovery of notexin and notechis II-5 in high purity.

6. A CROTOXIN-LIKE PROTEIN FROM THE VENOM OF A SOUTH AMERICAN RATTLESNAKE (CROTALUS DURISSUS COLLILINEATUS). Earlier work initiated on our first contract suggested that the venom of Crotalus durissus collilineatus contained a potent toxin. However, published studies by Faure and Bon (1987) reported that C. d. collilineatus venom contained only small amounts of a crotoxin-like neurotoxin in its venom. Subsequent fractionation of three different lots of collilineatus venom in

our laboratory demonstrated substantial amounts of a crotoxin-like protein in all three venoms. In fact, nearly 80% of the protein in one lot represented the crotoxin-like material, the highest percentage that we have observed in any rattlesnake venom to date. Many of its properties are similar to those of crotoxin, including its non-covalent heterodimeric structure, electrophoretic mobility on SDS-PAGE, isoelectric focusing properties, toxicity in mice, immunological reactivity, multiple isoforms, phospholipase activity, peptide map, and instability on an anion-exchange column. These results, reported in a manuscript by Lennon and Kaiser (1990), indicate that 'collilineatus toxin' is strongly homologous with crotoxin, found in the venom of C. d. terrificus, and all other characterized rattlesnake neurotoxins.

7. MOJAVE TOXIN SUBUNIT PREPARATION. We completed our preparation of highly purified acidic and basic subunits (20 mg each) for Dr. Keith Ward (Naval Research Laboratories, Washington, D.C.) using the published procedure of Aird and Kaiser (1985). Attempts at crystallization of these subunits are in progress in Ward's laboratory.

8. CROTOXIN SUBUNIT CROSS-LINKING. In work initiated under the last contract and recently completed, crotoxin was cross-linked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. Cross-linked crotoxin had the expected amino-terminal amino acids, amino acid composition, behavior on SDS-PAGE and 80% reduction of reactable lysine residues. It was also non-toxic, had reduced immunological cross-reactivity toward both poly- and monoclonal antibodies raised to the basic subunit of crotoxin and had lost >95% of its phospholipase activity. Loss of toxicity was due to either subunit cross-linking or the modification of essential residues. See the paper by Lennon, Plummer, and Kaiser (1990) for details.

9. X-RAY CRYSTALLOGRAPHY OF MOJAVE TOXIN. Refinement of the structure of Mojave toxin crystals in Ward's laboratory has been proceeding slowly. Details in the region of the unit cell containing the phospholipase subunit is well resolved, but the region containing the chaperone subunit is difficult to interpret and model building in this region is tenuous. As a result, despite extensive computational efforts using several different refinement programs (including molecular dynamics), the crystallographic R-value remains at 0.30. Substantial effort has therefore been spent at preparing new heavy atom derivatives in order to improve the current phase information, which should eventually lead to our understanding the structure.

Samples of acidic and basic subunit of Mojave toxin, as well as 5-15 mg samples of ammodytoxin, caudoxin, taipoxin, and paradoxin have been subjected to crystallization trials by Ward. Screens with ammonium sulfate, polyethylene glycol, and phosphate as precipitating agents have not yet resulted in crystals large enough for characterization by crystallographic methods.

b. MECHANISM OF ACTION.

1. CROTOXIN IODINATION AND BINDING STUDIES. Crotoxin has been iodinated using the chloramine-T method with both ^{125}I and ^{127}I, and extensively characterized. We obtain from 0.4-0.9 mol of ^{125}I per mol of intact crotoxin with a distribution of label between the basic:acidic subunits of 4:1. LD_{50}-values (i.v.) of ^{127}I-labeled crotoxin and sham-reacted crotoxin were 0.060 and 0.062µg/g in mice, respectively, with control crotoxin LD_{50}-values of 0.045µg/g. Phospholipase activity for native intact, native basic, ^{127}I-labeled, and mock-labeled crotoxin were determined to be 24, 85, 81, and 60 µmoles of L-α-phosphatidylcholine hydrolyzed min^{-1} mg^{-1}. In solid phase ELISAs, native, ^{127}I-labeled, and mock-labeled crotoxin all competed with biotinylated intact crotoxin for sites on each of the plate-bound monoclonal antibodies to crotoxin in a similar manner. Based on these studies, we feel that the iodinated crotoxin is similar enough to unmodified crotoxin to use in binding experiments.

Iodinated crotoxin was used to demonstrate high affinity, specific binding to guinea-pig (Cavia porcellus) brain, synaptosomal membrane fragments and to whole synaptosomes under a variety of conditions. ^{125}I-crotoxin binding to synaptosomal membrane fragments displayed two binding plateaus, (K_{d1} = 4 nM and K_{d2} = 87 nM, B_{max1} = 2, and B_{max2} = 4 pmoles/mg membrane protein), but binding to whole synaptosomes revealed only one plateau (K_d = 2 nM and B_{max} = 5 pmoles/mg membrane protein). Scatchard analysis yielded similar binding constants in the absence or presence of 0.025% Triton X-100, although non-specific binding increased in its absence. In addition to equilibrium analysis, kinetic analyses of ^{125}I-crotoxin binding to synaptosomal membrane fragments gave a K_d-value of 3 nM. Many presynaptic neurotoxins acted as inhibitors of crotoxin binding, which could be classified into three groups. The strongest inhibitory group included Mojave toxin, concolor toxin, taipoxin, paradoxin and pseudexin. Ammodytoxin a and textilotoxin were slightly weaker inhibitors, and notexin scutoxin A and notechis II-5 were the weakest of all. Non-neurotoxic phospholipases A_2 from C. atrox and

porcine pancreas showed little or no inhibition; nor did ß-bungarotoxin, dendrotoxin, and the post-synaptic neurotoxin notechis III-4. Addition of excess acidic, but not basic crotoxin subunit, was also inhibitory to crotoxin binding. Exclusion of added calcium lowered ^{125}I-crotoxin binding to synaptosomal membrane fragments, but did not significantly change the K_d-values or the number of binding sites. Treatment of synaptosomal membranes with protease K, but not trypsin, lowered ^{125}I-crotoxin binding. Binding of labeled crotoxin to erythrocytes was mainly non-specific. Iodinated, non-neurotoxic phospholipase A_2 from C. atrox showed no specific binding to synaptosomal membranes. These results suggest that crotoxin and several other presynaptic neurotoxins share common binding sites that may involve a protein on brain synaptosomal membranes. Details of this work have been described (Degn, Seebart, and Kaiser (1990); Degn, Seebart, Plummer, and Kaiser (1990).

2. ANTIBODY ESCAPE REACTIONS. In collaboration with Dr. Lance Simpson (Jefferson Medical College, Philadelphia), we have conducted studies on phospholipase A_2 neurotoxins and the cholinergic neuromuscular junction. A representative group of toxins have been examined, including notexin (one chain), ß-bungarotoxin (two chain, covalent association), crotoxin (two chains, non-covalent association), taipoxin (three chains) and textilotoxin (four chains). Experiments were done on the phrenic nerve-hemidiaphragm preparation. The purpose of the work was to determine whether the various snake neurotoxins undergo antibody escape.

The technique that was employed can be summarized as follows. Tissues were incubated with toxin at 4° C for 30 to 60 minutes, and then washed free of unbound toxin. Tissues were transferred to baths at 37° C, where phrenic nerves were stimulated and muscle twitch was recorded. At various times after transfer, tissues were exposed to neutralizing antibody. The purpose of the work was to determine how rapidly tissue-bound toxin would disappear from the neutralizing effects of antibody.

The data indicate that all multiple chain toxins disappear from accessibility to antibody, although at somewhat different rates. The single chain toxins have not yet been studied. The implication of the work is that the toxins insert into the membrane, or less likely that they cross the membrane.

In companion experiments, drugs that antagonize receptor-mediated endocytosis were tested as possible antagonists of snake neurotoxin. Thus far, none of these drugs has proved to be a universal antagonist. These findings may support the contention that snake neurotoxins do not

cross the cell membrane to exert their poisoning effect.

c. IMMUNOLOGY.

1. MONOCLONAL ANTIBODY CHARACTERIZATION AND SYNTHETIC PEPTIDE DESIGN. We have completed our examination of cross-reactivity of our four monoclonal antibodies and rabbit polyclonal antibodies raised against the basic subunit of crotoxin. Some of this work was described in my Final Report for contract no. DAMD 17-86-C-6061.

All four monoclonal antibodies appear to react with unique epitopes. Western blots on both non-reduced and reduced crotoxin samples, suggested that only one of the monoclonal antibodies(#11) partially reacted with reduced crotoxin. This indicated that it might at least partially recognize a continuous epitope. Octapeptides homologous with the complete sequence of the basic subunit of crotoxin were carried out on polyethylene pens according to the procedure of Geysen et al. (1987). These overlapping peptides were then used to screen our four monoclonal antibodies in an attempt to determine their antigenic determinants. Multiple assays failed to give consistent results, indicating that none of the four monoclonal antibodies recognized our solid-phase, linear epitopes. The same octapeptides were used to screen rabbit polyclonal antibodies. From the polyclonal assays, we identified four region from the basic subunit sequence that appeared antigenic. These were sequences 113-122 (SRSRGPSETC), peptide A; 102-115 (TYKYGYMFYPDSRC), peptide B; 31-44 (GGRGRPKDATDRSC), peptide C; and 1-9 (HLLQFNKMIC), peptide D. The highlighted S's in the 113-122 and 31-44 peptides have been substituted for a C. See Fig. 3 for the complete sequence of crotoxin subunits. These peptides were synthesized and coupled to carrier proteins (rabbit serum albumin) through their carboxy-terminus sulfhydral residues which naturally occurred in that position in peptides A, B, and C. An additional C was added to the C-terminus in peptide D for coupling purposes. Rabbits were immunized with conjugated proteins, boosted three times, and their antisera assayed for reactive antibodies.

2. ELISAS. Enzyme-linked immunosorbant assays (ELISAS) were carried out to determine the reactivity of polyclonal antisera raised against the four synthetic peptides conjugated to rabbit serum albumin. In addition, anti-peptide sera were screened for reactivity against free synthetic peptides, intact crotoxin, as well as its subunits.

Titers ranging from 1:250 to 1:160,000 were determined for the peptide antisera when assayed against their homologous, purified peptides conjugated to rabbit serum albumin (Fig. 1). None of the antisera reacted with rabbit serum albumin alone and only two sera, from rabbits

immunized with peptide B, showed reactivity when run against free homologous, non-conjugated peptide bound to the ELISA plate (not shown). Plate affinity for the free peptides may be a problem in some experiments, since in an assay using intact crotoxin antisera against both free and conjugated peptides, the conjugated peptides gave substantially better reactivity (not shown).

Antipeptide sera raised against three of the four peptides reacted well with intact crotoxin and its basic subunit (Fig. 2). Peptide D reacted most strongly in both assays, while C showed better reactivity than B. A did not react with either intact crotoxin or its basic subunit. None of the antipeptide sera reacted with the acidic subunit of crotoxin. This was not unexpected since the antigenic regions of the basic subunit identified by the Geysen procedure were either missing in the acidic subunit or had substantial amino acid sequence differences. For example, as shown in Fig. 3, peptide A had five amino acid differences out of nine; B had six amino acids missing in the corresponding acidic chain and six of eight of the remaining amino acids differed; peptide C was the most similar, with only four differences out of 14; and the entire peptide D region was absent in the acidic chain.

3. TOXIN NEUTRALIZATION WITH PEPTIDE ANTISERA. Antisera raised against each synthetic peptide were individually pre-mixed with 2 LD_{50}-doses of intact crotoxin before injection into mice. Neutralizing polyclonal antisera raised to intact crotoxin was combined with intact crotoxin in the same proportions and injected, serving as a positive control. A mixture of four antisera raised against each of the four different peptides was also pre-mixed with intact crotoxin and injected into mice. Only polyclonal antisera raised to intact crotoxin totally neutralized the intact crotoxin. Antipeptide sera raised against peptides B2 and B4 did delay the deaths of mice 1.5 to 2 times that of mice injected with intact crotoxin alone (see Table 1). Combined antisera pre-mixed with crotoxin also appeared to delay death in animals relative to controls (see Table 1).

4. F_{ab} FRAGMENTS. One of our aims in this contract was to prepare F_{ab} fragments of our neutralizing monoclonal antibody for crystallization attempts of the F_{ab}-antigen complex by Keith Ward. Ward obtained 14 mg crude monoclonal antibody directly from Dr. John Middlebrook (USAMRIID) and prepared F_{ab} fragments in his own laboratory. Some difficulty occurred in achieving complete digestion without long incubation times. Long incubation times produced a wide array of digested species, which then had to be purified prior to crystal growth experiments. Total digest

was initially purified by protein A affinity chromatography, which separated the F_{ab} fragments from the F_c region. DEAE-HPLC was used to purify the different species of Fab fragments produced from the digestion experiments. There were four major F_{ab} fragments with pI's of 5.3, 5.9, 6.5, and 7.2, all of which retained their ability to bind Mojave toxin as determined by dot ELISA. Complete purification of all four fragments was never completely attained. One of these fragments was hyperpurified to the point where it was >98% pure as determined by isoelectric focusing, and amounted to 1.2 mg of material. Crystal growth experiments conducted with this small sample have not yet been successful.

Low yields of purified F_{ab} is partially due to sample digest heterogeneity. With new samples of monoclonal IgG, Ward's laboratory intends to use shorter incubation times with soluble rather than immobilized papain to avoid this problem. Further, preparative isoelectric focusing will be used in an attempt to increase the yield and degree of purification of the F_{ab} samples. Since the purification of the F_{ab} fragments were initiated by Ward's laboratory, and they now have some experience with these preparations, my laboratory will do nothing further on this phase of the project until we hear further from Ward.

5. NATURAL RESISTANCE TO SNAKE VENOMS. Certain mammals and especially snakes are resistant to high doses of snake venom. This summer (1990) I have an undergraduate biochemistry student looking at the factor(s) responsible for the resistance of Crotalus atrox to its own venom. To date we have found that the factor is a serum protein with an apparent molecular weight of ca. 70 kDa. It does not appear to be an antibody and has many of the same properties of α_2-macroglobulins, except that it is only 1/10 the size. It is an inhibitor of venom protease activity and venom lethality. Attempts are continuing to isolate a purified factor from C. atrox plasma for further characterization.

d. MOLECULAR BIOLOGY.

1. CLONING. Messenger RNA was originally isolated from the venom gland of C. d. terrificus, and it was reverse transcribed. The product was amplified using the polymerase chain reaction and cloned into Bluescript II KS plasmids. Clones with inserts were partially sequenced, using the double-stranded method. All isolated clones were acidic subunit clones and of the six that were partially sequenced, all showed substantial sequence variability. It was unclear whether these represented different genes or whether we inadvertently introduced these base changes during

the amplification reaction. We decided to terminate our work on the C. d. terrificus materials and focus on cDNA and genomic libraries from the tissue of the Mojave rattlesnake (C. s. scutulatus), in collaboration with Dr. Leonard Smith (USAMRIID). Smith's laboratory has prepared the cDNA library, screened it for acidic and basic subunit genes, isolated several clones, and has completed sequencing three acidic subunit and four basic subunit clones. My laboratory is developing a strategy in an attempt to express these cDNA genes in an E. coli system. Smith is exploring expression of these genes in eucaryotic systems.

Total genomic DNA was prepared from C. scutulatus, type A liver as described by Davis et al., 1986. A kit from Promega Biotech was used to construct the genomic library using the manufacturer's protocols. C. scutulatus genomic DNA was randomly digested with Sau 3AI, under conditions to generate mostly large (=15-25 kb) DNA fragments. Overhanging ends of the insert DNA were then partially filled in with dGTP and dATP using Klenow fragment of DNA polymerase I. After initial reaction optimization, the appropriate ratio of insert and vector DNA were mixed, ligated using T4 DNA ligase, packaged into phage particles (lambda GEM-11 Xho I half-site arms from Promega), and used to infect host strain E. coli KW-251. About 3.6 x 10^5 plaque forming units (pfu) were obtained which was estimated to be a 95% representation of the total C. scutulatus genome. The library was amplified before screening with 42-base oligomeric DNA probes identical with the 5'-end of the basic subunit coding strand (5'-CACCTGCTGCAATTCAACAAGATGATCAAGTTTGAGACA AGG-3') and the 5'-end of the acidic subunit A-chain coding strand (5'-GGATGCTACTGTGGCGCGGGGGGCCAAGGCTGGCCACAGGAC-3'). About 150,000 pfu were screened with the above 42-mer probes and putative acidic and basic subunit clones identified. Three rounds of screening resulted in the purification of eight λ-DNA clones, with three positive for the acidic subunit, five for the basic subunit, and two hybridizing with both subunit probes. Insert size ranged from 7 to 16 kb.

Hybridization oligonucleotides complementary to DNA sequences immediately 5' (primer #1: 5'-TGCATGCCTGCAGAGACTTA-3') and 3' (primer # 2: 5'-CCCAGGTCTGGATTGAGGAGG-3') to the coding regions of both subunits of Mojave toxin were synthesized, and will be used as probes for subcloning. Preliminary hybridization experiments using the above probes suggest that five of the eight clones are truncated at the 5'-end of the toxin gene. Thus, we have one full-length putative acidic subunit gene clone, and two full-length putative basic subunit gene clones. Two of these three clones also hybridize with **both** of the 42-mer oligomers mentioned above. cDNA clones of both subunits of Mojave toxin provided by Dr. Leonard Smith, will also be used as hybridization probes

for genomic subcloning. These will insure that the genomic clones are not cleaved during the subcloning process.

2. IN VITRO CULTURING OF SNAKE VENOM GLAND CELLS FROM CROTALUS VIRIDIS CONCOLOR. We have been interested for some time in establishing a cell culture line of snake venom gland cells. Earlier unpublished work by Middlebrook suggested that it might be possible to establish such a line. More recently, Sells et al. (1989) reported the establishment of a long-term venom gland cell culture from Bitis gabonica. In collaboration with Kruger Bryant, Director of an on-campus tissue culture laboratory, we are attempting to establish a venom gland cell culture from glands removed from a young, C. v. concolor snake on June 27, 1990. Preliminary results indicate that the cells are still viable and are dividing--although slowly. Development of a viable venom gland cell line is critical for future studies of crotoxin subunit processing, as well as other venom production processes. The acidic subunit, is e.g. composed of three peptides which appear to be generated from a higher molecular weight precursor by several proteolytic cleavages. Identification and characterization of these processing proteases will require a viable tissue culture source of venom gland cells for raw material.

CONCLUSIONS

We have completed the sequencing of the basic subunit of Mojave toxin and the B-chain of crotoxin. This work completes the amino acid sequence studies on these two proteins, except for the determination of the disulfide bond arrangements within each subunit of the rattlesnake neurotoxins. We initially proposed a chemical determination for these, but now feel that these can best be determined by the x-ray analysis of Mojave toxin currently underway in collaboration with Dr. Keith Ward.

Amino acid sequences have been determined for two myotoxins from the venom of Bothrops asper. One, a potent phospholipase A_2 with 67% sequence identity with ammodytoxin a, represents the first myotoxin phospholipase sequenced that lacks presynaptic neurotoxicity. Domains and residues that may be responsible for neurotoxicity are suggested. The second phospholipase-like molecule sequenced is a lysine-49 phospholipase A_2 with key amino acid differences from active phospholipases. This work demonstrated that phospholipase activity *per se* is not required in phospholipase molecules for either myotoxicity or edema inducing activities.

We identified and have partially characterized additional notexin-like proteins from the venom of Notechis scutatus scutatus. These proteins

are immunologically related to notexin and notechis II-5 and have similar toxicities and phospholipase activities. Preliminary sequencing results indicate they are notexin isoforms.

In contradiction to earlier reports in the literature (Faure and Bon, 1987), we found up to 80% of the protein present in the venom of Crotalus durissus collilineatus to consist of a crotoxin-like protein.

Chemical cross-linking of crotoxin subunits with a water-soluble carbodiimide results in a loss of immunological cross-reactivity, phospholipase activity, and toxicity. We could not determine whether this was the the result of cross-linking directly or the modification of essential amino acid side chain residues.

Crotoxin iodination methodology has been developed and extensive binding studies have been carried out with the iodinated toxin on synaptosomes and their membranes. A high affinity (K_d = 2-4 nM), specific binding site was identified for crotoxin that could be strongly competed for by Mojave toxin, concolor toxin, taipoxin, paradoxin, and pseudexin. Ammodytoxin a and textilotoxin were less competitive with crotoxin binding and notexin, scutoxin A, and notechis II-5 even less. Non-neurotoxic phospholipases A_2 from C. atrox and porcine pancreas showed little or no inhibition; nor did ß-bungarotoxin, dendrotoxin, and the post-synaptic neurotoxin notechis III-4. Our binding results suggest that crotoxin and several other presynaptic neurotoxins share common binding sites that may involve a protein on brain synaptosomal membranes.

A series of experiments with Dr. Lance Simpson, employing structurally different snake venom presynaptic neurotoxins, have been carried out to determine whether neurotoxins undergo antibody escape. While the work is still in progress, preliminary results suggest that the toxins insert into the cell membrane, but do not cross the membrane, to exert their toxicity.

Studies designed to employ bioluminescence techniques to monitor continuous acetylcholine release from brain synaptosomes in vitro have been unsuccessful. Synaptosomes from brain tissues appear too "noisy" to use with this assay, apparently due to spontaneous acetylcholine release. We are investigating other biological properties of synaptosomes that could possibly be used to monitor effects that different toxins and other materials have on them, that could be used as a biological assay. One such property is choline uptake.

All four of our monoclonal antibodies raised against the basic subunit of crotoxin appear to react with unique, conformational epitopes. None of the four recognized any of the overlapping octapeptides, homologous with crotoxin's basic subunit sequence. In contrast, when rabbit polyclonal antibodies were screened in place of the monoclonals, four regions

appeared antigenic; the two end sequences plus internal regions corresponding to residues 31-44 and 102-115. Four peptides corresponding to these antigenic regions were synthesized, conjugated to rabbit serum albumin, and rabbits immunized. Resulting antisera failed to neutralized against crotoxin's lethality, although there was some extension of survival times (Table 1). In order to determine the epitopes of our monoclonal antibodies, we need to explore other available screening procedures. With the synthetic peptide antigens, we need to screen the literature for better carrier proteins.

cDNA clones for the acidic and basic subunits of Mojave toxin have been generated and sequenced by Dr. Leonard Smith. He has made these available to us and we are exploring their expression in E. coli systems. Our laboratory has isolated genomic clones for both subunit genes of Mojave toxin. These are currently being subcloned.

RECOMMENDATIONS

1. Determine the disulfide bond arrangements in Mojave toxin subunits from x-ray crystal structure studies now in progress.
2. Screen several additional Notechis and Pseudonaja subspecie venoms for previously unidentified neurotoxins. If any are found, carry out a preliminary isolation and characterization to determine their properties.
3. Sequence scutoxins A and B in collaboration with Dr. Jim Schmidt (USAMRIID).
4. Monitor results in Ward's laboratory on the (a) crystallization of the acidic and basic subunits of Mojave toxin, ammodytoxin, caudoxin, taipoxin, and paradoxin provided to him, (b) the preparation of F_{ab} fragments from our crotoxin neutralizing monoclonal antibody, and (c) determination of the phase angle in Mojave toxin crystals. Assist him by providing additional purified materials when available and if necessary.
5. Attempt to cross-link crotoxin to its synaptosomal binding site and do some initial characterization of that site, if possible.
6. Examine crotoxin interactions with nerve terminals in vivo using EM autoradiography.
7. Continue the antibody escape experiments with Simpson's laboratory.
8. Examine the potential of choline uptake by brain synaptosomes as a possible in vitro assay system for looking at presynaptic neurotoxin activity. Effects of different presynaptic and non-toxic phospholipases A_2 on uptake can be compared to determine if there is a correlation to their different potencies.

9. Once procedures are located for the elucidation of conformational epitopes, these should be applied to our monoclonal antibodies.

10. If a better carrier protein for our synthetic peptides can be identified, we will conjugate it to our synthetic peptides, immunize additional animals, and screen the resulting antisera for neutralizing antibodies.

11. An undergraduate student should continue to work on the isolation and characterization of the protein present in snake sera that is able to inhibit venom protease activity and venom lethality.

12. Attempt to develop an expression system in E. coli for the acidic and basic subunit cDNA clones of Mojave toxin. Smith is attempting expression in eucaryotic systems.

13. Continue with the subcloning of the Mojave toxin subunit genomic DNAs. Once subcloned, initiate sequencing.

14. Continue the collaboration with Kruger Bryant on the in vitro culturing of snake venom gland cells from C. v. concolor. If established, it may be possible to carry our some preliminary biochemical and immunological experiments with these cells.

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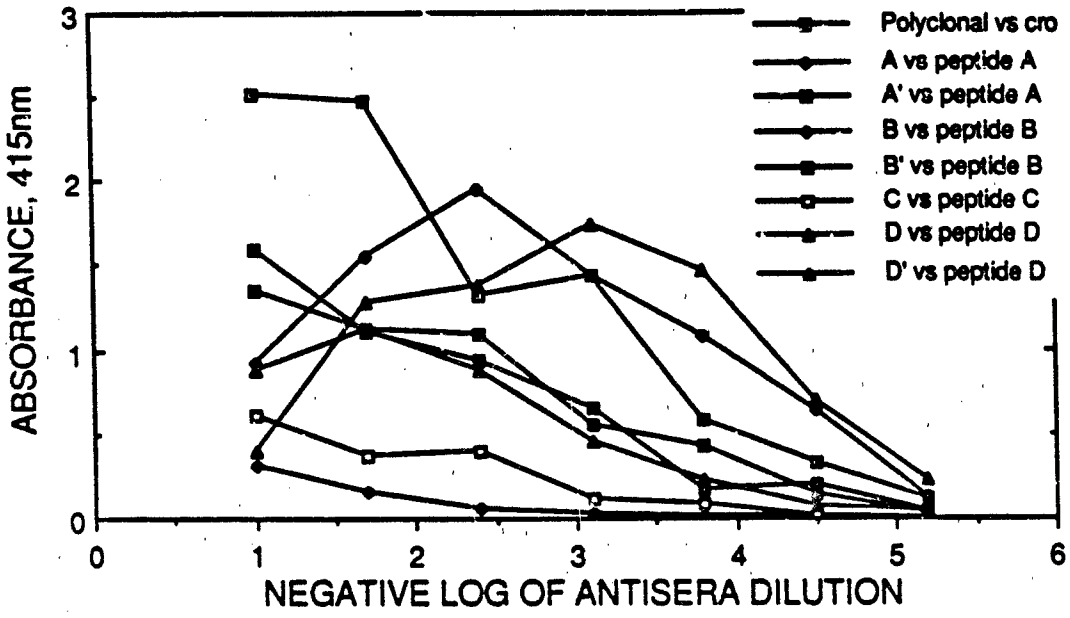

Fig. 1. ELISA titrations of rabbit antisera raised against intact crotoxin and four different peptides, as listed to the right of the figure, and reacted with their homologous antigens. Peptides A, B, C, and D correspond to sequences 113-122, 102-115, 31-44, and 1-9, respectively, in the basic subunit of crotoxin. These were conjugated to rabbit serum albumin as described in the text and two rabbits were immunized with each conjugate. Plates were coated with homologous antigen and serially diluted rabbit sera assayed for reactivity.

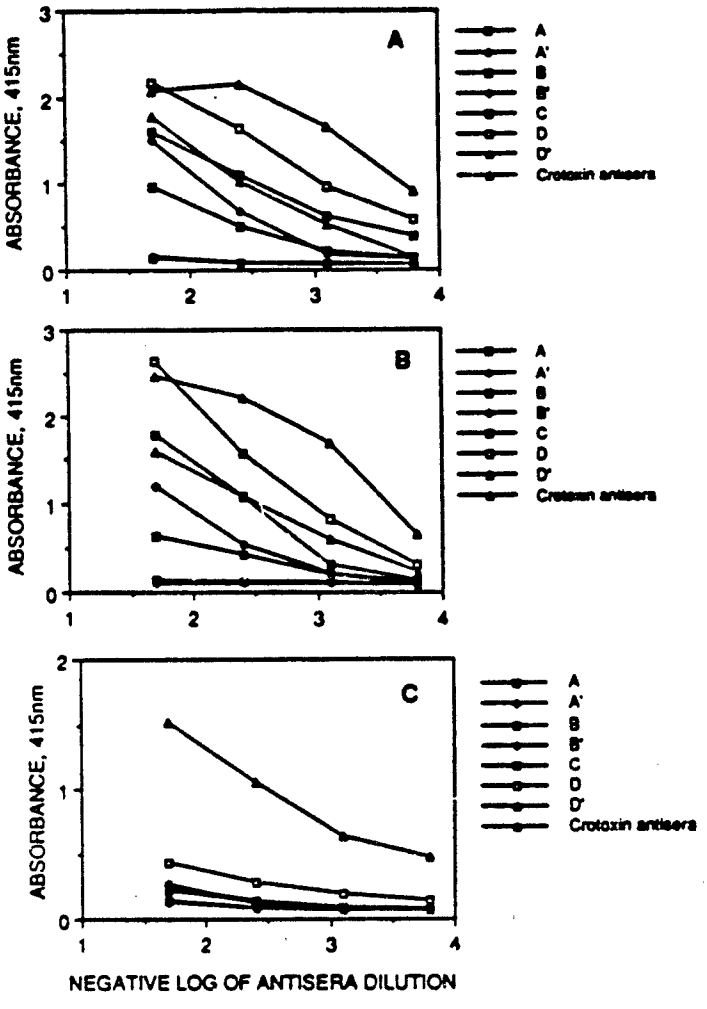

Fig. 2. ELISA cross-reactions with intact crotoxin (A), and its basic (B) and acidic (C) subunits. Plates were coated with either intact crotoxin or one of its subunits and ELISAs were carried out with rabbit antisera raised against the antigens listed to the right of each figure.

ACIDIC AND BASIC SUBUNITS OF CROTOXIN

Taxon	Protein	5	10	15	20	25	30
C. d. terrificus	Crotoxin Acidic	. . . . .	. . . . .	. . . . .	. . . S Y G C Y C G	A
C. d. terrificus	Crotoxin Basic	H L L Q F N K M I K F E T R K N A I P F Y A F Y Q C Y C Q	W

		35	40	45	50	55	60
C. d. terrificus	Acidic	G G Q G W P Q D A S D R C C F E H D C C Y A K L T Q C N P	T
C. d. terrificus	Basic	G G R G R P K D A T D R C C F V H D C C Y G K L A K C N T	K

		65	70	75	80	85	90
C. d. terrificus	Acidic	. . . . . pE E D G E I V C G E D D P C Q T Q I C Q/E C D	K
C. d. terrificus	Basic	W D I Y R Y S L K S Q Y I T C Q K Q T W C E E Q I C E C D	R

		95	100	105	110	115	120
C. d. terrificus	Acidic	A A A I C F R N S M D T . . . . . pE F S P E N C Q Q E S	Q
C. d. terrificus	Basic	V A A E C L R R S L S T Y K Y Q Y M F Y P D S R C R Q P S	E

C. d. terrificus	Acidic	P C
C. d. terrificus	Basic	T C

Fig. 3. Amino acid sequence of the acidic and basic subunits of crotoxin.

Table 1. PEPTIDE ANTIBODY PROTECTION AGAINST LETHALITY

		SURVIVAL TIMES (HOURS) MALE MICE*								
ANTISERA	ANTIGEN	< 1	> 1 < 2	> 2 < 3	> 3 < 6	> 6 < 24	> 24			No. injected
A	Peptide B1		2	2		1				5
A'	Peptide B1		1	1	1	1				4
B	Peptide B2				2	2				4
B'	Peptide B2			2	1	1				4
C	Peptide B3	1		3						4
D	Peptide B4				1	3				4
D'	Peptide B4			1	4					5
T24	Crotoxin						4			4
None				4						4
		FEMALE MICE*								
A+B+C+D		1				4				5
None			1		2	1				4

Table 1. Neutralization experiments consisted of pre-mixing 2µl crude antisera with either 0.1 or 0.13µg intact crotoxin per gram of male or female mouse, respectively, before i.v. injection. Four individual antisera raised against peptides A-D were combined, mixed with crotoxin (0.9µl individual sera or 3.5µl total combined antisera were mixed with 0.13µg intact crotoxin per gram of female mouse), and injected i.v. into mice. LD_{50} value for crotoxin is 0.05µg/g in male and 0.065µg/g in female mice.